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Comorbidity between Alzheimer's Disease and Seizure Episodes

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Abstract

A mouse model of Alzheimer's disease (AD), which overexpress the human amyloid precursor protein (APP), experience Kainic Acid (KA) induced seizures that are more severe than wild type (WT) mice. The latency and severity of seizures observed in AD mice are similar to those of Synaptophysin knockout (Syp KO) mice. Addition of amyloid plaques (Aβ) to cells in vitro leads to the breakdown of Syp-VAMP2 complex. In the present study, we investigated if the breakdown of Syp-VAMP2 complex is responsible for the observed seizure activity in AD mice. The binding of cholesterol to Syp is necessary for biogenesis of synaptic vesicles, and cholesterol-depleted cells have reduced Syp-VAMP2 complex. Being deficient in cholesterol, we expected to observe seizure susceptibility and also reduced Syp-VAMP2 complex in Apolipoprotein E4 (ApoE4) mice as well. We tested WT, AD, ApoE4, and Syp KO mice for susceptibility to KA induced seizures. Our findings revealed that AD, ApoE4, and Syp KO mice all have similar seizure severity and latency. Using western blot analysis we examined the levels of Syp-VAMP2 complex present in the brains of these mice at 6 months but we failed to observe a difference. We are currently devising more sensitive methods for measuring the abundance of the Syp-VAMP2 complex in the brain.

Introduction

There are a number of different diseases that compromise brain function. However, the exact mechanisms of malfunction are not always transparent. Alzheimer's disease (AD) is a neurodegenerative ailment, characterized by gradual but, severe loss of cognition, including memory and intellectual skills (Cedazo-Mínguez, 2007). AD is the most common form of dementia in people over 65 years of age (Cedazo-Mínguez, 2007; Van Spronsen & Hoogenraad, 2010). Sporadic AD is the most common form of the illness; with unclear primary causes. Nonetheless, aside from aging, a combination of susceptibility genes and environmental factors could contribute to its development (Cedazo-Mínguez, 2007). Apolipoprotein E4 (ApoE4) is the primary risk factor for sporadic AD (Hirsch-Reinshagen, Burgess, & Wellington, 2009). Familial AD, the less common form of the disease, distinctly develops, due to mutations in the amyloid precursor protein (APP) and presenilins genes (Cedazo-Mínguez, 2007).

The notion of synaptic dysfunction is a well recognized phenomenon in AD (Näslund et al., 2000; Tampellini et al., 2010; Van Spronsen & Hoogenraad, 2010). Therefore, the synaptic function abnormalities observed in AD may be better addressed as an independent phenomenon or as the primary symptom of AD, rather than an event subsequent to neuronal loss. The two hallmark pathologies of AD are known to be amyloid plaques (Aβ) formation outside the neurons and neurofibrillary tangle formation within the neurons, which are primarily consisted of tau proteins (Cedazo-Mínguez, 2007; Hirsch-Reinshagen et al., 2009; Stoothoff & Johnson, 2005). Despite extensive research, a successful prevention/treatment for the disease has not been discovered (Sutton et al., 2015).

Tau Hypothesis

Tau protein stabilizes microtubules by becoming phosphorylated. Hyperphosphorylation (3-4 folds higher than normal levels) of tau however, disrupts its normal function of axonal transport regulation and also leads to the formation of neurofibrillary tangles, which could cause synaptic dysfunction and eventually cell death (Lloret, Fuchsberger, Giraldo, & Vina, 2015; Stoothoff & Johnson, 2005).

Aβ ***Hypothesis***

Aβ is a peptide ranging from 36 to 43 amino acids, produced as a result of proteolytic cleavage of amyloid precursor protein (APP) by β- or γ secretase (Zhao et al., 2012). The major components of Aβ are 40 and 42 amino acids long peptides (Aβ40 and Aβ42 respectively). These peptides easily build up and harden to form insoluble oligomers. The oligomers further accumulate and give rise to Aβ, which cause synaptic dysfunction between neurons (Charkhkar et al., 2015). While the exact processes through which Aβ deposits contribute to neuronal damage remains elusive, the patients clinical condition appears to be strongly correlated with the composition of Aβ (Murphy &

LeVine, 2010). Studies done on primate brains have given rise to the hypothesis that Aβ fibrils could also promote peculiar tau phosphorylation (Hardy, Duff, Hardy, Perez-Tur, & Hutton, 1998).

Neurotransmission

Action Potential

The release of neurotransmitter upon action potential arrival, its propagation down the cell membrane, and the subsequent synaptic terminal depolarization is a synchronized multistep process. This intricate event requires complex machinery, made up of numerous proteins that mediate efficient membrane fusion and neurotransmitter release (Jena, 2011).

Neurotransmitter Storage/Release

Classical neurotransmitters are stored in small and clear vesicles that release their content in a Ca^{++} activity-dependent manner (Sudhof & Jahn, 1991), where an influx of calcium ions into the nerve terminal through voltage–gated calcium channels leads to the fusion of vesicles to the plasma membrane and neurotransmitter discharge into the synaptic cleft (Feany, Yee, Delvy, & Buckley, 1993; Fykse et al., 1993; Jena, 2011).

Vesicle fusion

Vesicle fusion to the target membrane is mediated by SNARE proteins, an abbreviation for SNAP receptor [soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein], including Synaptobrevin, which is localized in synaptic vesicles (SVs), as well as Synatxin and SNAP 25 that reside in the plasma membrane (Becher et al., 1999; Gordon & Cousin, 2014; Cho, Jeremic, Jin, Ren, & Jena, 2007). During this process, vesicular proteins or v-SNAREs interact with target proteins or t-SNAREs, followed by the vesicles being recycled back from the plasma membrane through endocytosis (Jena, 2011). Being an exothermic process, SNARE complex assembly releases the energy that functions as a fuel during the fusion process (Wiedelhold & Fasshauer, 2009). Following exocytosis, the conformation of trimeric complex changes from *trans* to *cis* and it is disassembled to monomers by NSF, which functions as an ATPase, rendering them free to participate in another round of fusion. At this point, Synaptobrevin returns to the SV membrane and Synataxin and SNAP 25 to the plasma membrane (Otto, Hanson, & Jahn, 1997; Südhof, & Rizo, 2011). The function of SNAREs were discovered through the function of clostridial botulinum and tetanus toxins, which act as proteases and chop up SNARE proteins and consequently inhibit neurotransmitter release by blocking SVs membrane fusion to the plasma membrane (Südhof et al., 2011). Membrane trafficking processes including, docking, priming, fusion, exocytosis and/or endocytosis of vesicles are possible by a complex interaction of numerous SV proteins; among them is the integral membrane protein, Synaptophysin (McMahon et al., 1996).

Synaptophysin

Synaptophysin 1 (Syp) is a transmembrane glycoprotein that is found in small presynaptic vesicles of nerve and neuroendocrine cells (Kwon & Chapman, 2011; Reisinger et al., 2004). Syp is the most abundant synaptic vesicle membrane protein, making up of ~ 10% of the total SV proteins by mass (Kwon & Chapman, 2011). Syp was the first SV protein to be isolated and cloned. Syp has a 4 transmembrane domain and is the most heavily tyrosine phosphorylated protein in the SV (Evans & Cousin, 2007). Study of Syp in SV dynamics, eventually lead to the discovery of other SV proteins and since then, efforts have been made to uncover the specific role that each of these proteins play in membrane trafficking (Valtorta, Pennuto, Bonanomi, & Benfenati, 2004). Despite being known for so long, a clear function has not yet been assigned to Syp. It has been suggested that it is involved in synaptic functions such as exocytosis, biogenesis (Li, Reinprecht, Fahnestock, & Racine, 2002), synaptogenesis, and endocytosis of SVs (Kwon & Chapman, 2011).

Syp KO and Normal Neurotransmission

Even though Synaptophysin is the most abundant vesicle membrane protein, the Syp KO mice do not show an observable phenotype. It seems that the synaptic vesicle exocytosis remains efficient in Syp KO mice, as revealed in their normal neurotransmission (Becher et al., 1999; Kwon & Chapman, 2011; Evans & Cousin, 2007). This phenomenon may be due to redundant expression of other Syp isoforms, Synaptoporin (Synaptophysin II), and Synaptogyrin (Janz et al., 1999; Kwon & Chapman, 2011) Despite the fact that Syp KO mice have no observable phenotype, double knockout mice (lacking SypI/Synaptogyrin I) show clear deficits in synaptic plasticity and LTP (Kwon & Chapman, 2011) but they seem to have normal neurotransmitter release (Valtorta et al., 2004). This suggests that Syp may not be essential for synaptic activity, but perhaps functions to modulate vesicle cycle efficacy (Schmitt, Tanimoto, Seeliger, Schaeffel, & Leube, 2009).

SYP-VAMP2 Complex and Endocytosis

Syp and Synaptogyrin I are suggested to be strong regulators of exocytosis (Janz et al., 1999). Syp has also been suggested to be required for regulating endocytosis of the SVs. This idea was further supported by examination of the retinal rod photoreceptors morphology in in Syp KO mice (Arthur & Stowell, 2007), as they don't express Synaptoporin (Synaptophysin II) and have SV endocytosis deficiency, which seems to result from reduced number of SV, following prolonged high frequency firing (Wu & Wu, 2009). Additionally, Syp KO neurons exhibit defective SV endocytosis in vitro, while exocytosis and the size of the total recycling pool of SVs remain unaffected, which further supports the role of Syp in regulating rapid and efficient SV endocytosis and also correlates with the pronounced depletion and slower recovery of synaptic vesicles in Syp

KO neurons (Kwon & Chapman, 2011). Although Syp does not appear to be essential for endocytocis, it seems to be required for increased endocytosis efficiency, as its presence leads to effective SV retrieval, following continued stimulation and drastic synaptic depression is observed in its absence (Kwon & Chapman, 2011) as timely and efficient recycling of SVs is necessary for their maintenance in the active zone (Evans & Cousin, 2007).

Syp binds to Synaptobrevin (vesicle-associated membrane protein, or VAMP2), a member of v-SNAREs to form Syp-VAMP2 complex, necessary for exocytosis of synaptic vesicles. This complex has been suggested to contribute to SV fusion efficiency by speeding up the process, since frequent stimulation of kindled rats has been correlated with increased levels of this complex (Reisinger et al., 2004). The main function of Syp has been suggested to be contributed to endocytosis process, by returning VAMP2 back into SV membrane (Adams, Arthur, & Stowell, 2015). It has been suggested that Syp's main role is regulating synaptic responses by controlling the availability of Synaptobrevin to form SNARE complex. The binding of Syp to Synaptobrevin prevents Syntaxin and SNAP 25 to attach to Synaptobrevin and provides a rapidly available pool of Synaptobrevin to fuse with membrane during exocytosis (Becher et al., 1999; Mitter et al., 2003), since once the complex is formed, Synaptobrevin would require the NSF-mediated disassociation, in order to be able to participate in exocytosis (Becher et al., 1999). Syp is also known to bind to cholesterol. Although, it is not yet clear what function this interaction serves (Adams et al., 2015), it is assumed to promote biogenesis of synaptic-like microvesicles in neuroendocrine cells (Evans and Cousin, 2007). Remarkably, excess levels of Aβ and decreased levels of cholesterol have both been associated with disruption of Syp-VAMP2 complex (Arthur & Stowell, 2007).

ApoE4 and Seizures

In central nervous system, Apolipoprotein E (ApoE) is produced by astrocytes and is responsible for transporting cholesterol to neurons (Cedazo-Mínguez, 2007; Poirier, 2000). ApoE gene has three different alleles in humans, with ApoE3 being the default isophorm and present in about %78-80 of population, the ApoE2 being a protective factor for AD and present in about 8% of the population, and ApoE4, the most important risk factor for AD pathogenesis, being present in 14–16% of individuals (Pfrieger, 2003; Zhong & Weisgraber, 2009). ApoE4 is a poor transporter of cholesterol. Even though at least one copy of ApoE4 allele is present in 40-65% of AD patients, about 33% of AD patients do not have the ApoE4 allele and not all ApoE4 homozygotes develop AD (Hauser & Ryan, 2016). This by no means discounts the importance of the role ApoE4 plays is AD pathogenesis (Pfrieger, 2003). It is not clear as to how this isoform contributes to the development of AD, since it is involved in a wide array of biochemical pathways (Cedazo-Mínguez, 2007), but it has been suggested to play an important role in safeguarding and repair of neurons, by distributing the necessary lipids

necessary for their proliferation and synaptogenesis. ApoE4 has also been suggested to modify Aβ deposition (Cheng, Zhou, Holtzman, & Han, 2010) and post-mortem studies of the AD patient with this allele have been shown to have more Aβ deposits (Fu et al., 2010). There is correspondingly a correlation in literature between cholesterol homeostasis, Aβ formation and secretion, and ApoE metabolism (Poirier, 2000). ApoE4 has also been suggested to be a susceptibility gene for epilepsy and its presence seems to be correlated with increased incidents of posttraumatic seizures and earlier onset of Temporal Lobe Epilepsy (TLE). Increased Aβ deposition has also been observed TLE patients (Fu et al., 2010).

AD and Seizures

Primary literature suggests that there is comorbidity between seizures and AD in humans (Miranda, Maria, & Brucki, 2014), however, the actual underlying mechanisms remain obscure (Vossel et al., 2013). About 10% to 22% of patients with AD experience seizures. Nevertheless, the accuracy of this data is questionable, as the AD patients' sample size studied are usually small and varied in illness severity. Additionally, since dementia is a clinical symptom of the illness, the occurrence of seizures in AD patients could potentially be underreported. But seizures are believed to occur prior to cognitive decline (Vossel et al., 2013). Types of seizures that AD patients experience are rarely reported in the literature, although partial seizures seem to be more frequently encountered in this population (Miranda et al., 2014).

The preliminary data previously obtained in our lab also suggests that that AD mice have seizures that are more severe than the wild type mice, indicating that from a physiological point of view, there is a correlation of the comorbidity of seizures and dementia in mice as well. This data also shows an association between AD mice and Syp knock out mice, as their seizure phenomenon is clearly distinguished from the WT mice. We were curious to know if there is comorbidity between ApoE4 transgenic mice and seizures, and if the loss of Syp is related to the seizure phenomenon. Our proposed mechanism is that the binding of Aβ to Syp disrupts the Syp-VAMP2 complex and thus, interferes with synaptic vesicle fusion and subsequent neurotransmitter exocytosis. This connection was shown in previous in vitro experiments in our lab, where the addition of Aβ42 drastically inhibited neurotransmitter release by preventing the catalytic functions of Syp and consequently, its binding to VAMP2 (Adams et al., 2015). However, this study has not been done in vivo. There is a correlation in literature between the loss of Synaptophysin and AD (Bash, 2015). We aim to further study this connection through both behavioral and molecular approaches in order to investigate if ApoE4, Syp KO, and AD mice have a similar seizure profile and if they do, is they have a similar and detectable profile of Syp-VAMP2 Complex. Despite the lack of any reported phenotype in Syp KO mice, it has previously been observed that Syp KO mice experience unprovoked seizures. Initial experiments were done and the preliminary data obtained in

our lab suggested that the latency and severity of seizures in AD mice corresponds to that of Syp KO mice. To our knowledge, the seizure susceptibility of ApoE4 mice has never been pharmacologically studied before.

Experimental design

Our primary manipulation and independent variable was the use of transgenic mice comprising of Synaptophysin Knock out mice (Syp KO), Alzheimer's model mice (APP/PSEN), and Apolipoprotein Epsilon 4 mice (APOE4), which we believe to share commonality in seizure frequency (severity and latency). We attempt to demonstrate this theory by inducing Kainic Acid (KA) seizures in transgenic mice, which is assumed to cause excessive epileptogenic activity in excitatory glutamatergic circuitry (Heggli & Malthe-Sorenssen, 1982), in order to reduce the threshold of seizure. The progression of seizures were evaluated using Racine scale and the latency was measured based on the time period required for a mouse to reach each seizure stage, within 60 minutes time frame. To determine the presence and quantity of Syp-VAMP2 complex, synaptosomes of untreated mice brains of age 7-9 months of age with different genotypes were prepared and the complex was pulled down via co-immunoprecipitation**.**

Methods

Behavioral Analysis

The mice were weighed and injected intraperitoneally (IP) with 25mg/kg Kainic acid monohydrate (purchased from sigma, St. Louis, MO), diluted with Phosphate buffered saline (PBS) using Insulin syringe. The control mice were injected with PBS only. The mouse was placed in a 15cm radius observation bucket, filled with its own bedding material for the mice comfort.

The mice were monitored and recorded right away for 60 minutes, unless culminated in death before a full hour was up. A canon camera was focused on the mirror, reflecting the mouse in the observation bucket. Seizure intensities (severity) was measured using the 8-stage Modified Racine Scale (Butler et al., 1995) and the latency was measured by the time it took a mouse to reach a certain seizure stage. It is important to note that not each seizure stage has to be reached before developing higher seizure levels. The observations were performed blind and the genotypes of the mice were only known to the person doing the injections masked form the person monitoring the mice. Figure 1 shows the settings in which mice were injected, observed and recorded.

All mice (male and female) were 3-6 months of age. A total of 9 WT mice, including 5 previous WT mice data obtained by our lab [(C57BL/6J, Jackson Laboratory, Bar Harbor, Maine, USA)] were injected with KA, plus 4 controls that were injected with PBS only (3 mice were excluded, due to KA measurement errors). A total of 4 ApoE4 mice [B6.Cg-Tg (GFAP-APOE i4) 1Hol *Apoe*^{tm1Unc}/J Jackson Laboratory, Bar Harbor, Maine, USA] were injected with KA, plus 2 controls that were injected with PBS only. 4

AD model mice [B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J Jackson Laboratory, Bar Harbor, Maine, USA) and a total of 5 Syp KO mice (a kind gift from Dr. Rudolf Leube, University of Aachan, Germany) were also injected with KA, with no controls (gathered from preliminary data). Mice were sacrificed by cervical dislocation following the experiment and the brain was rapidly frozen in liquid nitrogen and kept frozen at -80.

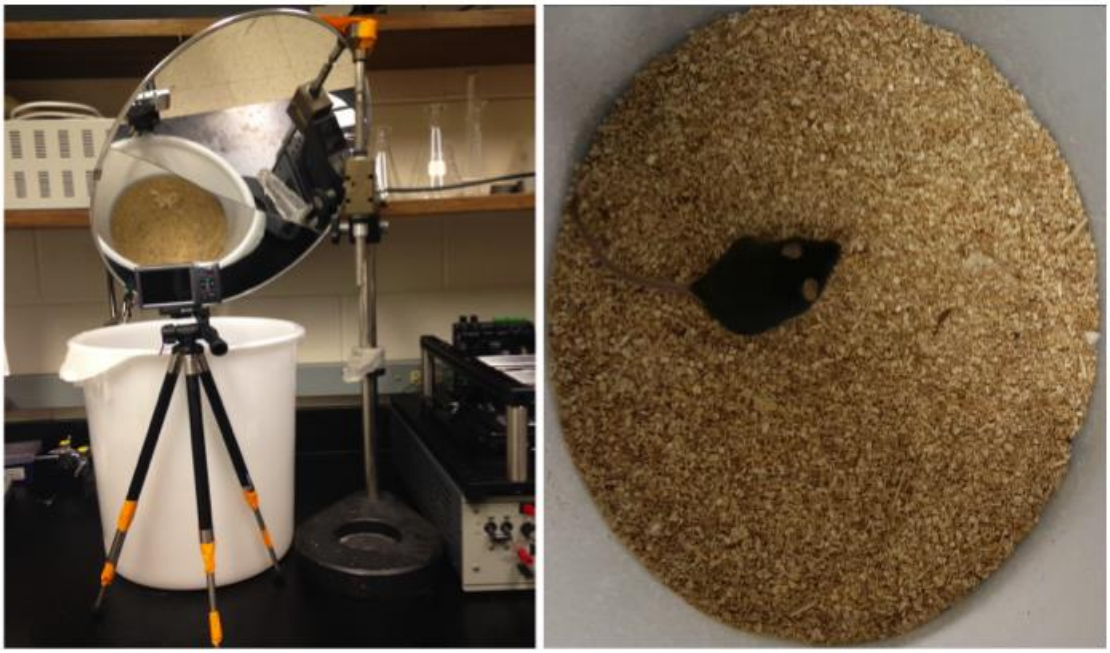

Figure 1. Testing/observation Room. The mice were monitored and recorded for 60 minutes, in a 30 cm radius bucket, following 25mg/kg KA IP injection.

Table 1: Modified Racine Scale used to measure severity of epileptiform activity in mice

Racine Scale	**Behavior Observed**
0	Normal Exploratory Behavior
1	Immobility, Facial Clonus
2	Head Nodding
3	Unilateral Forelimb Clonus
4	Bilateral Forelimb Clonus
5	Rearing and limb clonus with loss of postural control
6	Running And Bouncing
7	Tonic Hindlimb Extension
8	Tonic Hindlimb Extension Culminating In Death

Molecular Analysis

Crude Synaptosomal Preparation

Whole adult mouse brains of 6 months-old WT, ApoE4 and AD mice were homogenized on ice in 3 mL sucrose buffer (0.32M), with 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.1 mM EDTA (pH 8.0) and Protease Inhibitor Cocktail Complete (Roche), by 20 up and down strokes with a glass homogenizer. Homogenous material was transferred to a 15 mL conical tube and kept on ice. A further 2 mL of sucrose buffer was added to any remaining brain and the brain was homogenized by a further 20 up/down strokes. Homogenous material was pooled and centrifuged at 1,000 x G for 10 minutes at 4°C, in order to pellet the brain tissue debris, following homogenization. The supernatant was removed and stored on ice and the pellet re-suspended in 3 mL of homogenization buffer and centrifuged at 1,000 x G for 10 minutes at 4°C. Supernatants were pooled and poured into "high-speed centrifuge proof" tubes and centrifuged at 20,000 x G for 15 minutes at 4°C, in order to pellet intact and lysed synaptosomes. The supernatant was discarded and the pellet (crude synaptosomes) homogenized/re-suspended thoroughly in 2 mL of sucrose buffer (with inhibitors) with 0.1%Triton X100 and kept on ice for approximately 45 minutes, mixing periodically. The lysate was centrifuged at top speed on a bench top centrifuge at 4°C for 20 minutes and the pellet discarded leaving the supernatant. Aliquots of the sample were taken at this stage. Lysates were cleared with 20 µL of Protein G-agarose (Roche) for 1 h at 4°C (under rotation). Following which, beads were pelleted at 3,000 rpm for 1 min at 4°C and supernatants transferred to a new 1.5 mL eppendorf tube. Aliquots of the samples were taken as "Post-Clear" input at this stage. Lysates (containing equal amounts of protein following BCA assay) were made up to 1 mL in homogenization buffer. 10 µL (2 µg) of anti-Syp primary antibody (SVP 38, mouse monoclonal IgG_1, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the 1 mL of lysate and incubated for 1 h at 4°C under rotation. 30 µL of Protein G-agarose was added to the lysate/antibody mix and incubated overnight at 4 °C under rotation. Beads were spun down at top speed at 4°C and the supernatant discarded. Beads were 5 times washed in 1mL 0.32 M Sucrose buffer (ice cold) with 0.1% Triton at 4°C on under rotation, for 5 minutes. After each wash, beads were spun down at top speed for 1 min and the supernatant discarded (the first wash supernatant was collected as a control). For the final wash, the entire volume of sucrose buffer was removed with gel loading tip, in order to leave dry beads. Beads were re-suspended in 50 µL of 2X SDS loading buffer with βME and heated at 95°C for 10 minutes. Beads were vortexed and spun down and collected to a new tube. Input samples were prepared from the Pre- and Post-Clear lysates.

Co-Immunoprecipiation

The supernatants were loaded on polyacrylamide gels (4-15% gradient gel) at 100V, 50 mA, and 50W for 1 hour. The bands were transferred onto nitrocellulose. The blot was blocked for 1 h in Tris-Buffered Saline and Tween 20 (TBST) and 5% dry non-fat milk. The membrane was cut at about 25kDa. The membrane smaller than 25kDa was incubated in anti-VAMP2 antibody (Rabbit Polyclonal Antiserum - 1:1000 in in 1x TBST/5% NFDM - Synaptic Systems, Göttingen, Germany) and the one larger than 25kDa was incubated in anti-Syp primary antibody (SVP 38, mouse monoclonal IgG_1, Santa Cruz Biotechnology, Santa Cruz, CA, USA - 1:2000 in 1x TBST/5% NFDM). The membranes were incubated overnight at 4 °C. The membranes were washed 3 times with TBST for 5 minutes. Membranes were then incubated in secondary antibodies that were diluted in TBST in a 1:10,000 ratio for an hour. VAMP2 was detected with secondary antibody goat anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Syp was detected using goat anti-mouse (Santa Cruz biotechnology, Santa Cruz, CA, USA). The membranes were washed 3 times with TBST for 5 minutes and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was applied. The membranes were exposed on x-ray film for 5 minutes and then developed accordingly.

Results

Behavioral

All wild type mice had a maximum of R2 (immobility and head nodding) KA induced seizures, except for one that reached R6 (rearing and limb clonus with loss of postural control) during the experiment. Up to R8 (tonic hindlimb extension culminating in death) seizures were observed only in ApoE4, AD and Syp KO mice and not in WT mice. The P values of each genotype verse all others were calculated and shown in table 2 for R6 and table 3 for R8. In order to present a more clear evaluation, the survival curve of each genotype was plotted (instead of latency vs. severity graph) for R6 and R8, in figures 2 and 3 respectively. The reason for choosing R6 was that it is absolutely distinguishable from R8, and from every Racine scale below R6. R6 and R8 provide a clear demarcation among seizure levels. The data clearly shows a similar survival ratio between transgenic mice and their distinguished seizure susceptibility from WT mice.

Table 2: P values of survival between each two mice genotype in R6-Seizure: There was no significant difference in survival rate between Syp KO vs. AD, Syp KO vs. Apoe4, and AD vs. ApoE4 in R6. The survival rates between WT and transgenic mice were statistically significant.

Mice Genotype	P value	Mice Genotype	P value
WT vs. Syp KO	<0.002	Syp KO vs. AD	0.9
WT vs. AD	<0.005	Syp KO vs. ApoE4	> 0.1
WT vs. ApoE4	<0.01	AD vs. ApoE4	0.21

Table 3: P values of survival between each two mice genotype in R8-Seizure: There was no significant difference in survival rate between Syp KO vs. AD, Syp KO vs. Apoe4, and AD vs. ApoE4 in R8. The survival rates between WT and transgenic mice were statistically significant.

Mice Genotype	P value	Mice Genotype	P value
WT vs. Syp KO	0.0001	Syp KO vs. AD	0.05
WT vs. AD	0.0001	Syp KO vs. ApoE4	0.025
WT vs. ApoE4	0.04	AD vs. ApoE4	0.16

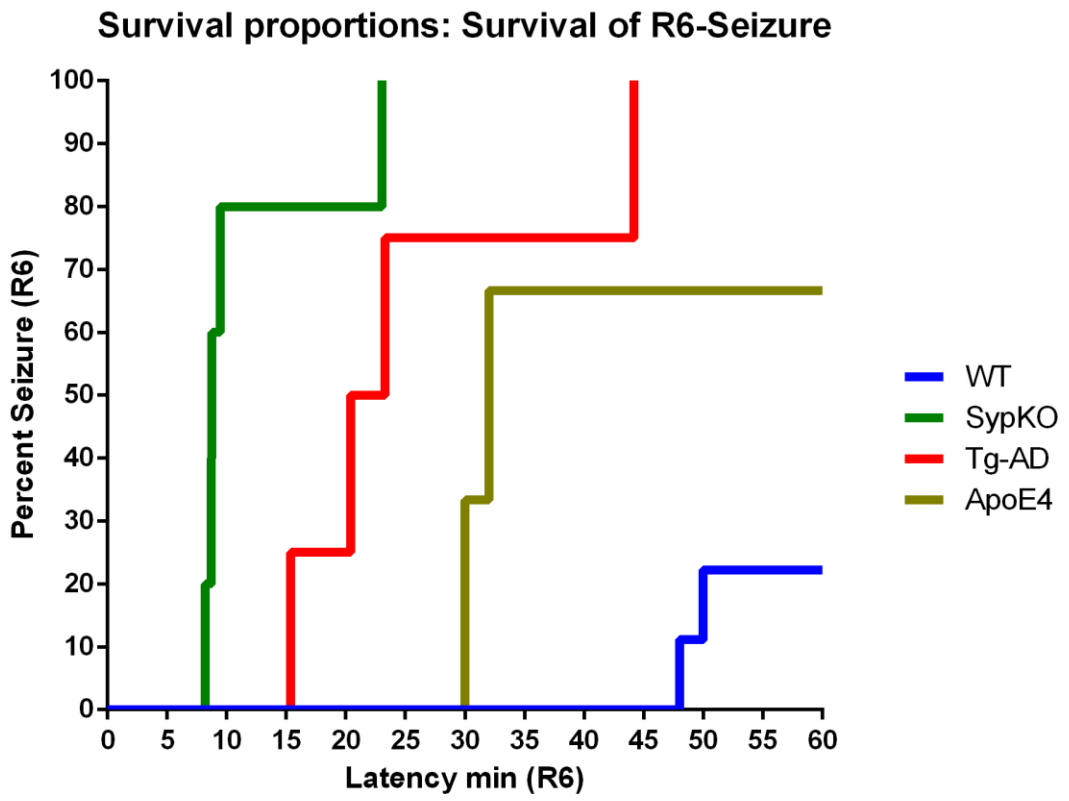

Figure 2: Survival Curve for R6-Seizure after Kainic Acid seizure induction. The graph shows that transgenic mice have a similar survival rates at R6, whereas WT mice had a significantly different survival rate, based on their latency in reaching R6-Seizure.

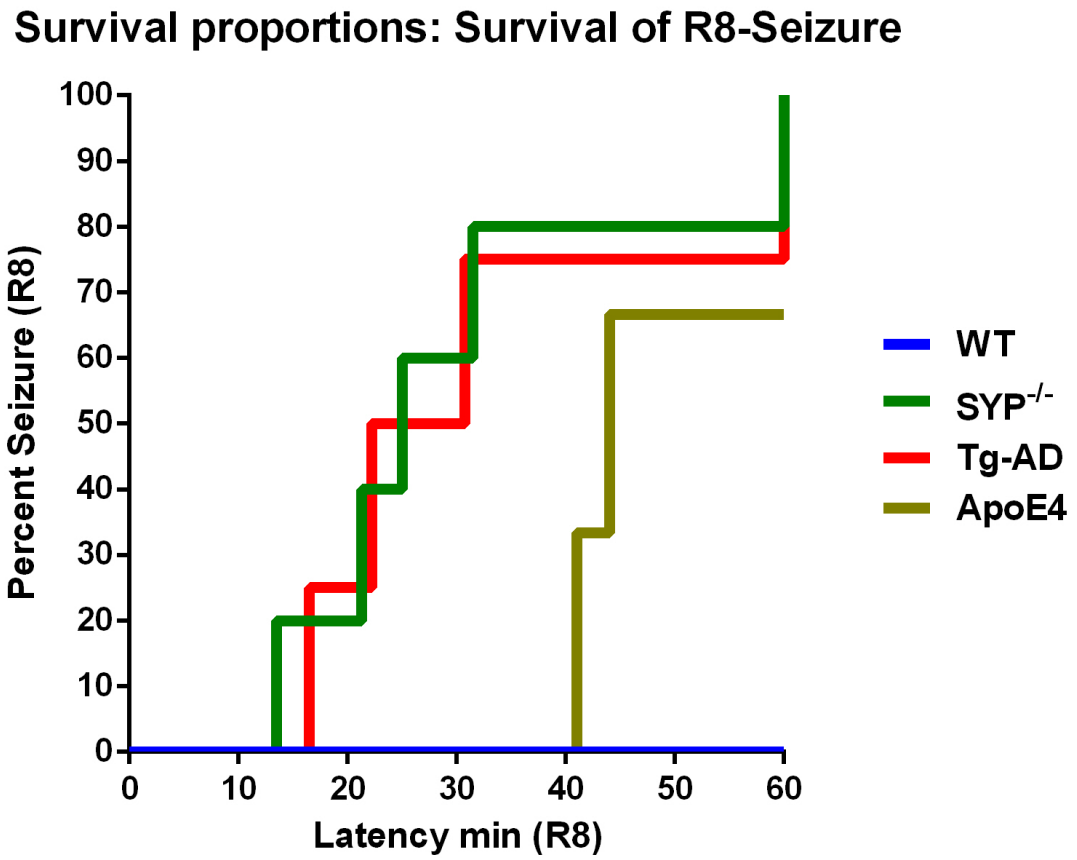

Figure 3: Survival Curve for R8-Seziure after Kainic Acid seizure induction. The graph shows that transgenic mice have a similar seizure profile at R8, whereas no WT mice reached this seizure stage.

Molecular

Our molecular data did not indicate a difference in the Syp-VAMP complex amount among WT, Apoe4 and AD mice. We captured VAMP2 and looked for Syp in the complex. Different quantities of sample were loaded (5, 10, 15 µL), with the expectation to get equal VAMP2 captured, based on which Syp levels could be evaluated. Our molecular data shows there to be more Syp in AD mice compared to WT and demonstrates no a detectable difference between AD and Apoe4 mice.

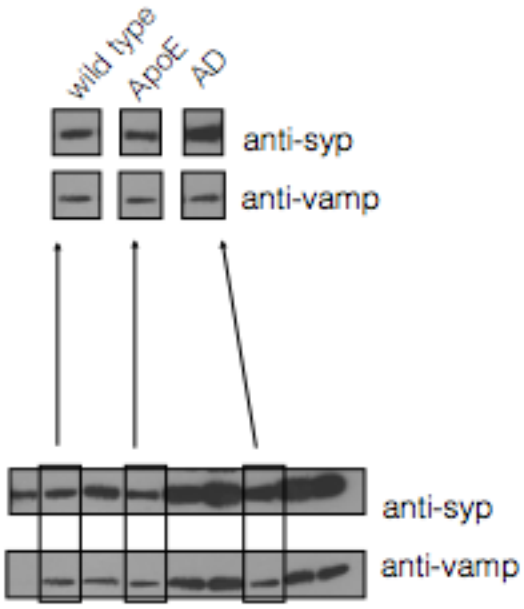

Figure 4: Western blot analysis of Syp-VAMP2 complex in 6 month-old WT (5, 10, 15 μL), ApoE4 (5, 10, 15 μL), and AD mice (5, 10, 15 μL). VAMP2 was captured to detect Syp in the complex. VAMP2 rabbit polyclonal antibody as primary and goat anti-rabbit as secondary antibody were used for VAMP2 detection. SVP 38 mouse monoclonal as primary and goat anti-mouse as secondary antibody were used for Syp detection. There was no significant difference in Syp-VAMP2 complex abundance in ApoE4, AD and WT mice. However, AD mice brain showed a slightly more intense band for Syp. Syp bands were observed at 32kDa and VAMP2 at 17kDa.

Discussion

Syp KO, ApoE4 KO and the AD model mice had similar KA induced seizure phenotypes. However, reduction in the abundance of the Syp-VAMP2 complex was not observed. The lack of observable reduction in the Syp-VAMP2 complex in these genotypes could reflect our expectation that this phenomenon is only happening in readily releasable pool (RRP) vesicles, which are docked at the active zone. This is presumably the case for Aβ interactions with SVs, where only RRP vesicles that are going through turnover are subject to Aβ-mediated modifications and not either the reserve or the recycling pool. It could also be the case that he loss of Syp-VAMP2 complex in this age group is not detectable by western blot method, since it is not known how much complex loss is necessary for seizures to take place. Re-doing the molecular analysis in older AD mice may provide more information to uncover this phenomenon. We also expected to see relatively lower amounts of complex in ApoE4, compared to the WT mice, as cholesterol is not absolutely eliminated in this genotype and some complex could still be present. Examining older ApoE4 mice and comparing them to age-matched WT could help us make better conclusions, assuming that ApoE4 mice seizure phenotype also deteriorates with age (Snyder et al., 2005).

AD and Syp KO

Normal brain function is dependent on coordinated synaptic function to provide efficient neurotransmission (Van Spronsen & Hoogenraad, 2010). Synaptic deficiency in AD patients is currently the strongest link to cognitive decline (Abramov et al., 2009). The underlying mechanisms of seizures in AD are not yet clear, but exposure to pathologically relevant levels of Aβ induces hyperexcitability in individual neurons and neural circuits (Miranda et al., 2014). The gradual loss of Syp is also confirmed in AD transgenic mice (Tampellini et al., 2010) and our preliminary data shows that the presence of Aβ disrupts Syp-VAMP2 complex. Knocking out the SYP gene leads to seizures, as it has been observed in our lab. Our current behavioral study shows a clear similarity in severity and latency of KA induced seizures in ApoE4, AD (APP) and Syp KO mice and we postulated the breakdown of Syp-VAP2 complex to be the underlying mechanism responsible for the hyperexcitability phenotype.

The normal neurotransmission observed in Syp KO mice has been suggested to be due to the actions of other compensatory paralog proteins (Janz et al., 1999; Kwon & Chapman, 2011). The only structural alterations reported in Syp KO mice has been reported to be a reduction in Syp-VAMP2 complex (McMahon et al., 1996) and mice lacking both Syp and the related tetraspan proteins of the same family have deficient synaptic plasticity, but not a significant impairment in neurotransmission (Evans & Cousin, 2007), which may be due to compensatory mechanisms established during development (Becher et al., 1999).

Although Synaptophysin and Synaptoporin have a significant sequence similarity, they don't have the same subcellular localization and the distribution of Syp is more homogenous than Synaptoporin (Brandstatter, Lohrke, Morgans, & Wassle, 1996; Fykse et al., 1993), which could reflect that Syp may serve a more specific function (McMahon et al., 1996).

We believe there to be no difference in seizure severity and latency in Syp KO mice in different age groups, due to the absence of Syp from birth. However, we have not tested Syp KO mice seizure severity across different age groups, in order to evaluate the presence of any compensatory mechanisms earlier or later in life. Seizures observed in AD mice, however appears to be more severe in early stages (Born, 2015). Immunoreactivity studies in AD transgenic mice have reported that the accumulation of intraneuronal Aβ happens prior to plaque formation (Tampellini et al., 2010), long before the loss of synapses and neurons (Van Spronsen & Hoogenraad, 2010) and the appearance of cognitive symptoms in AD mice (Venkitaramani et al., 2009).

Neurotropic vs. Neurotoxic Effects of Aβ

Loss of neuromodulatory effect of Aβ could lead to overexcitement

Studies by Roberto Malino have shown that Aβ production is enhanced, as a result of neuronal activity and that increased formation of Aβ leads to depression of

excitatory synaptic transmission (Venkitaramani et al., 2009). According to recent studies and contrary to traditional hypothesis, optimal and yet, precise amounts endogenous Aβ seem to function as neuromodulatory factor and are necessary for normal synaptic function, by controlling the neurotransmission release probability. Disruption of this fine balance could result in synaptic irregularity and dysfunction (Abramov et al., 2009). The amount of Aβ produced, released and degraded is observed to be positively correlated with synaptic activity (Abramov et al., 2009; Van Spronsen & Hoogenraad, 2010). This phenomenon led to the hypothesis that in a normal brain, Aβ may function as a neuromodulatory factor that keeps synaptic transmission in check and prevents overexcitation by being increasingly produced along with increased synaptic function (Abramov et al., 2009; Venkitaramani et al., 2009). According to this view, then it is expected to see excess Aβ production, as a result of overexcitation and epileptic activity. However, epilepsy is not a known risk factor for AD development. It is also important to note that seizure incidents are not limited to AD patients and they could also occur in conditions such as, strokes and head injury. Remarkably, traumatic head injury is a known risk factor for AD development and is believed to trigger a transitory elevation of Aβ levels in the brain (Born, 2015; Schroeder & Koo, 2005).

Additionally, neurotransmitter release probability in excitatory synapses appear to be highly sensitive to immediate changes in Aβ levels, compared to that of inhibitory synapses (Abramov et al., 2009). It may be that the accumulation of Aβ may not solely a result of faulty APP cleavage, but rather due to vesicle release mechanisms that result in activity-dependent variations in synaptic Aβ levels (Schroeder & Koo, 2005) and that epileptic activities observed in early AD are a consequence of synaptic failure (Van Spronsen & Hoogenraad, 2010). Interestingly it has been suggested that both increased or decreased level of Aβ could result in diminished vesicle release (Abramov et al., 2009).

Although modification of Syp expression in neurons remains obscure, it has been suggested that Siah proteins, encoded by SIAH gene have an important role neuronal function and synaptic function by ubiquitin-dependent degradation specific proteins, including Syp. Siah is suggested to have a role in Syp expression regulation by ubiquitinating and degrading it as its overexpression results in reduced Syp expression and this process is only feasible when Syp in not in a complex with VAMP2 (Wheeler, Chin, Li, Roudabush, & Li, 2002).

There has not yet been a biochemical experiment, which directly evaluates the function of Syp in SV fusion but given that Aβ appears to be released synaptically (Schroeder & Koo, 2005) to function as a neuromodulator of synaptic activity in a normal brain (Abramov et al., 2009) and that its excessive levels could elicit epileptiform activity in vivo, prior to cognitive decline (Miranda et al., 2014) in initial stages of the disease (Vossel et al., 2013), it would be reasonable to assume that the interaction of Aβ with Syp may lead to faulty neurotransmission that leads to overexcitaton and seizure phenomenon by primarily, the lack of neuromodulatory effects of Aβ on neurotransmission and

consequently, the termination of Syp's regulatory effect on SV trafficking. So, by preventing the synaptic response modifying mechanisms exerted by Syp, which are induced by Aβ neuromodulatory activity, the emergence of overexcitation is a reasonable expectation. On the other hand, chronic synaptic inhibition appears to reduce levels of Syp and the number of synapses (Tampellini et al., 2010), this could reflect a reciprocal cause and effect leading to further loss of efficient synaptic function that eventually leads to neuronal loss. Reduced occurrence of seizure activities in late AD could further support this idea.

Aβ has also been suggested to lessen the neuronal threshold to insults such as, overactivity induced by excitatory amino acids, glucose deficiency and oxidative stress (Näslund et al., 2000). In recent studies it has also been shown that glutamate uptake by glial cells is disrupted by Aβ administration and could result in toxic glutamatergic overactivity (Harkany et al., 1999). Aβ accumulation could also further lead to hyperexcitability by increasing resting Ca^{++} concentrations within neurons (Fu et al., 2010).

ApoE4

ApoE4 mice have dysfunctional astrocytes, unable to fully support and maintain neurons and as the animals age, they become more and more inefficient to counteract cellular insults, such as oxidative damage and Aβ toxicity. Moreover, levels of the astrocyte-specific glutamate transporter, GLT1 have also been observed to decrease in ApoE4 mice, which could lead to inefficient glutamate clearance and excitotoxicity (Zhong & Weisgraber, 2009). Also, the fact that inhibition of cholesterol synthesis in the brain could significantly affects Aβ production provides another mechanism via which, ApoE4 could contribute to Aβ accumulation(Poirier, 2000). ApoE has been suggested to function as Aβ scavenger and its concentration is inversely correlated with that of Aβ, whereas ApoE4 is devoid of this function (Poirier, 2000), which could further contribute to the pathogenicity of the disease. It is notable that despite these observed correlations, seizure susceptibility of AD patients has not been yet evaluated in relation to ApoE4 genotype. In ApoE4 and AD mice, the lack of or reduced complex formation might be due to an undetermined modification of Synaptophysin (Becher et al., 1999).

Cognitive impairments in AD

Synaptic function is crucial to cellular and molecular mechanisms of learning and memory, which inevitably depends on normal neurotransmission (Bash, 2015). The gradual process of cognitive decline observed in AD is likely an independent symptom that arises from loss of synapse and neuronal death as the disease progresses. Moreover, recycling of Aβ to the cell surface after APP internalization results in signaling cascades on post synaptic membrane that resemble those observed in LTD such as, AMPA receptors internalization and reduced spine on dendrites (Van Spronsen & Hoogenraad,

2010), which could further result in cognitive impairments observed in AD. However, Patients with AD and seizure disorders have greater cognitive impairment, faster progression of symptoms, and more severe neuronal loss at autopsy than those without seizures (Vossel et al., 2013).

Conclusion

We believe that the shared phenotype of seizures experience observed in ApoE4, Syp knock out and AD mice is linked to Syp-VAMP2 complex abundance in the brain. Although, specific role of Syp in synaptic transmission is barely understood, the presence of a shared phenotype in transgenic mice, as well as future evaluations of the Syp-VAMP2 complex levels may suggest a novel link to better understanding the pathology of AD and also providing a synaptic marker that foreshadows, and potentially prevents the development of the disease. We believe that the molecular methods employed for the purpose of our study might offer limited detection and the employment of techniques such as, proximity ligation assay in future studies may provide a better tool for quantitative measurements of the Syp-VAMP2 complex levels.

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